In vivo Comet assay: Additional comments on intra- and inter-laboratory reproducibility

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In the 3rd phase pre-validation and 4th phase validation stages of the JaCVAM collaborative trial of the in vivo Comet assay, i.e. at stages where a robust protocol had been developed, the intra- and inter-laboratory reproducibility shown by the direct-acting positive control chemical, ethyl methanesulfonate (EMS), and also by methylnitrosourea (MNU) was very good. However, the reproducibility shown by 2-acetylaminofluorene (2-AAF), the positive control chemical requiring metabolism, was poor. Since the dose levels of 2-AAF used in the three laboratories participating in Phase 4-1 were not similar, the VMT considered that it would be difficult to assess the consistency of assay results in those three laboratories that tested 2-AAF. Finally, the VMT considered that 2-AAF was judged as inconclusive due to the variable testing conditions. Therefore the VMT decided that 2-AAF would be re-examined in Phase 4-2 of the validation study under the coded test chemical conditions. In the one laboratory that tested 2-AAF in Phase 4-2 of the trial, negative results were obtained both in liver and stomach. Other chemicals that require metabolic activation gave expected positive results (e.g. 2,4-diaminotoluene, N-nitrosodimethylamine), however, each was only tested in a single laboratory and therefore intra- and inter-laboratory reproducibility could not be established. There may be some concerns that the in vivo Comet assay does not give reproducible responses with chemicals that require metabolic activation.

It was therefore decided to try to obtain additional data for the in vivo Comet assay to assess the intra- and inter-laboratory reproducibility of chemicals requiring metabolic activation. Two approaches were taken:

- Industrial laboratories were asked to provide any in-house data on testing of positive control chemicals known to require metabolic activation

- Data from the published literature (in particular the publications of Rothfuss et al, 2010 and Bowen et al, 2011 – papers appended) were reviewed.

Data from industrial laboratories

Letters were sent to a number of laboratories requesting data, and replies with data were received from seven different companies. These data are summarised in Appendix 1. It can be seen that:

- DMN was positive in the liver in 1 laboratory, but since it was also positive as a coded chemical at a similar dose in Phase 4-2 of the JaCVAM trial, it can be considered that acceptable inter-laboratory reproducibility has been achieved.

- Cyclophosphamide (CP) was positive in the liver in 1 laboratory, but was negative in another and showed inconsistent results in the liver in a third laboratory. Given the evidence that CP is effectively detoxified by GSH conjugation, it might be expected that it would not consistently produce DNA damage in the liver. Comets might only be detected when detoxification by GSH has been saturated.
• Acrylamide was positive in the liver in 1 laboratory. Since it was positive according to statistical analysis in Phase 4-2 of the JaCVAM trial, it can be considered that acceptable inter-laboratory reproducibility has been demonstrated.

• DMBA was positive in the liver in 1 laboratory when sufficiently high doses were tested. However, as it appears it was only tested once, and was not included in the JaCVAM trial, intra- and inter-laboratory reproducibility cannot be concluded from this.

• Aflatoxin B1 was tested in 1 laboratory and gave a positive response, but this was associated with histopathological changes and so the result was inconclusive. However, as it appears it was only tested once, and was not included in the JaCVAM trial, intra- and inter-laboratory reproducibility cannot be concluded from this.

• N-butyl-N-(4-hydroxybutyl)-nitrosamine was tested in 1 laboratory and gave positive responses at the same dose levels in both liver and urinary bladder in males and females of 3 different strains of rat. Therefore, intra-laboratory reproducibility was clearly demonstrated with this chemical.

The additional data supplied by the 7 laboratories on compounds not requiring metabolism (i.e. EMS, MMS, MNU and AZT) that were also included in the pre-validation and validation phases of the JaCVAM trial, further demonstrate the inter-laboratory reproducibility of the assay.

Data from Rothfuss et al (2010)

The following compounds expected to require metabolic activation were evaluated in the studies presented in this paper:

• benzo[a]pyrene (B[a]P) – not carcinogenic in liver, but induces DNA adducts and transgenic mutations in rodent liver
• 1,2- dimethylhydrazine (1,2-DMH) – liver carcinogenicity not tested, but induces DNA adducts, comets and UDS in rodent liver
• 2,6-dinitrotoluene (2,6-DNT) – liver carcinogen and induces DNA adducts and UDS in rodent liver
• dimethylnitrosamine (DMN) – liver carcinogen and induces DNA adducts, comets, UDS and transgenic mutations in rodent liver
• 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) – liver carcinogens and induces comets and transgenic mutations in rodent liver
• 2,4-diaminotoluene (2,4-DAT) – liver carcinogen and induces UDS and transgenic mutations in rodent liver
• acrylamide (ACR) – not a liver carcinogen but does induce DNA adducts and comets in rodent liver, although it is negative for UDS and transgenic mutations.

In addition 1,2-dibromomethane (1,2-DBE), which is a liver carcinogen in females only, and induces comets and UDS in liver after ip administration, but does induce DNA adducts in liver, was included. Also, 2,6-diaminotoluene (2,6-DAT), which is a non-carcinogenic analogue of 2,4-DAT, and three non-genotoxic liver carcinogens (methapyrilene (MP), clofibrate (CFB) and phenobarbital (PHE)) were included.

Male rats received oral administrations of the test compounds, daily for two or four weeks. The top dose was meant to be the highest dose producing clinical signs or histopathological effects without causing mortality, i.e. the 28-day maximum tolerated dose. The liver Comet assay was performed
according to published recommendations and following the protocol for the ongoing JaCVAM validation trial. Laboratories provided liver Comet assay data obtained at the end of the long-term (2- or 4-week) studies together with an evaluation of liver histology. Although each chemical was tested in a single laboratory (except for IQ which was tested in 2 laboratories), most of the test compounds were also investigated in the liver Comet assay after short-term (1–3 daily) administration to compare the sensitivity of the two study designs.

The key results can be summarised as follows:

- 1,2-DMH was positive in liver in both acute and repeat-dose protocols. It was also positive in liver in Phase 4-2 of the JaCVAM trial.
- 2,6-DNT was positive in liver in both acute and repeat-dose protocols.
- DMN was positive in liver in the repeat-dose protocol. It was not tested in the acute protocol. It was also positive in liver in Phase 4-2 of the JaCVAM trial.
- 1,2-DBE was positive in liver in both acute and repeat-dose protocols. It was also positive in liver in Phase 4-2 of the JaCVAM trial.
- IQ was positive in liver in the repeat-dose protocol and positive in 1 of 2 laboratories in the acute protocol.
- ACR was positive in liver in both acute and repeat-dose protocols. It was also positive in liver (according to statistical analysis) in Phase 4-2 of the JaCVAM trial.

B[a]P did not induce liver comets in either the acute or repeat-dose protocol. However, it is not a liver carcinogen. 2,4-DAT also did not induce liver comets in the repeat-dose protocol but was not tested with the acute protocol, which did produce a positive response in the JaCVAM trial. On the other hand, 2,6-DAT, which might have been expected to give negative results, was positive in liver in both acute and repeat-dose protocols. However, this is consistent with published findings (see Rothfuss et al, 2010 for references) and results in the JaCVAM trial.

These data therefore demonstrate acceptable intra-laboratory reproducibility (between acute and repeat-dose protocols in the same facility), demonstrate acceptable inter-laboratory reproducibility (in comparison with published findings and the JaCVAM trial), and demonstrate the applicability of the assay for detecting liver genotoxins that require metabolic activation.

Of the 3 non-genotoxic liver carcinogens, all were negative for comets in liver using the repeat-dose protocol. However, PHE induced liver comets with the acute protocol, as has been described in the published literature (see Rothfuss et al, 2010 for references). These data also demonstrate the applicability of the assay in giving negative results with chemicals expected to be non-genotoxic in liver.

**Data from Bowen et al (2011)**

The following compounds, expected to require metabolic activation were evaluated in the studies presented in this paper:

- 2-acetylaminofluorene (2-AAF) – administered ip
- benzo[a]pyrene (B[a]P) – administered ip
- cyclophosphamide (CPA) – administered orally
- dimethylnitrosamine (DMN) – administered orally

Male rats were dosed at 0, 24 and 45 h, and liver, whole blood and stomach were sampled for comet analysis three hours after the last dose. The Comet assay was performed according to published recommendations. In addition, bone marrow and peripheral blood were sampled at the same time for micronucleus evaluation.
The key results can be summarised as follows:

- **2-AAF** induced comets in liver but not in stomach or blood when administered ip. It had given negative comet responses in all tissues when administered orally in initial trials. These effects are consistent with those seen in the JaCVAM trial.
- **B[a]P** induced comets in stomach but not in liver or blood. It had given negative results in all tissues when administered orally in initial trials, which is consistent with results obtained in the JaCVAM trial, and by Rothfuss et al (2010).
- **CPA** induced comets in stomach and blood but not in liver. The negative results in the liver were not clearly explained, but may be due either to efficient detoxification of reactive metabolites or to possible DNA crosslinking mediated by the metabolite phosphoramide mustard. The induction of comets in stomach may have been mediated via another pathway, or acid hydrolysis (due to the environment of the stomach) may have yielded a non-crosslinking mutagen.
- **DMN** induced comets in liver and blood but not in stomach. These results are consistent with those obtained in the JaCVAM trial and by rothfuss et al (2010).

Thus, overall, there is good reproducibility for the detection of comets in liver and stomach by compounds requiring metabolism both within and between laboratories. These data complement the reproducibility demonstrated for direct-acting carcinogens and mutagens in the JaCVAM pre-validation and validation trials.
## Appendix 1: Data from 7 laboratories

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Compounds tested</th>
<th>Study design</th>
<th>Results</th>
<th>Overall call</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMS</strong></td>
<td>CP (10mg/kg/d)^a</td>
<td>1-month Oral Integrated Pig-a, MN, and Comet assay. Male Sprague-Dawley rats. 150 Nuclei analysed per sample. Comet IV image analysis. Rats were dosed with all 3 positive controls by either PO or ip route on the days indicated: (a)Days 1-2, 27-28 with CP by PO route; (b)Days 1-3 with ENU by ip route; (c)Day 30, 3 hr before necropsy with MNU by PO route.</td>
<td>Significant increase in DNA damage in liver cells</td>
<td>Positive</td>
<td>Brain (EMS) and blood (MNU) data also available (po)</td>
</tr>
<tr>
<td></td>
<td>ENU (10mg/kg/d)^b</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>MNU (2.5mg/kg/d)^c</td>
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<tr>
<td><strong>Merck</strong></td>
<td>EMS 200 mg/kg (PO) [1X and 2X]</td>
<td>Followed JaCVAM protocol. 1X = rats dosed once, 3hrs prior to necropsy; 2X = rats dosed twice 23 and 3hrs prior to necropsy</td>
<td>Significant increase in DNA damage in liver cells</td>
<td>Positive</td>
<td>Results provided for different vehicles. Here presented overall results. Stomach data also available (positive, increase in DNA damage)</td>
</tr>
<tr>
<td><strong>DMN 2.5mg/kg (PO)</strong></td>
<td>Followed JaCVAM protocol.</td>
<td>Significant increase in DNA damage in liver cells</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Acrylamide (PO) 50 mg/kg</strong></td>
<td>Followed JaCVAM protocol.</td>
<td>Significant increase in DNA damage in liver cells</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ILS (NTP data)</strong></td>
<td>Cyclophosphamide</td>
<td>MN/Comet studies (4 days of dosing) for the NTP that either tested CP, or utilized CP as a positive control. Followed JaCVAM protocol but there were some differences in electrophoresis temperature, however it does not change the overall case based on concurrent controls</td>
<td>CP didn’t reliably test positive in the liver, but it did in other organs (leukocytes and duodenum). Data published, Recio et al 2010 J. Toxicol. Sci Vol35, No.2, 149-162. Raw data was provided.</td>
<td>Equivocal</td>
<td>One of the debates on CP is cross links vs. GSH detoxication for lack of response for CP. ILS showed that it is effectively detoxified by GSH conjugation.</td>
</tr>
<tr>
<td><strong>BioReliance</strong></td>
<td>DMBA 2.5, 5, 10 mg/kg</td>
<td>29 days oral administration. Integrated Pig-a, MN, and Comet assay. Samples collected 3-4 hours after last dose. Followed JaCVAM protocol.</td>
<td>No increase in DNA damage in liver cells</td>
<td>Negative</td>
<td>Study repeated at higher doses</td>
</tr>
<tr>
<td></td>
<td>DMBA 10, 50, 100 and</td>
<td>Acute dosing. Samples collected 3-4 hours</td>
<td>Increased DNA damage in liver</td>
<td>Positive</td>
<td></td>
</tr>
</tbody>
</table>

^a^ Followed JaCVAM protocol. 150 Nuclei analysed per sample. Comet IV image analysis. Rats were dosed with all 3 positive controls by either PO or ip route on the days indicated: (a) Days 1-2, 27-28 with CP by PO route; (b) Days 1-3 with ENU by ip route; (c) Day 30, 3 hr before necropsy with MNU by PO route.

^b^ Followed JaCVAM protocol. 1X = rats dosed once, 3hrs prior to necropsy; 2X = rats dosed twice 23 and 3hrs prior to necropsy.

^c^ Followed JaCVAM protocol.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose/Method</th>
<th>Groups</th>
<th>Data Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mg/kg</td>
<td>after last dose. Followed JaCVAM protocol</td>
<td>cells</td>
<td>No increase in DNA damage in liver cells</td>
</tr>
<tr>
<td>J&amp;J CP</td>
<td>Single oral dose oral; liver of male SD rats; tissue sampling at 3 and 24 hour post-dose; Saline was used as vehicle control; No histopathology was conducted; Top dose of 30 mg/kg was not the MTD, but was based on published data (Ashby and Beije, MR 150 (1985): 383-392) demonstrating that dose levels of &gt; 30 mg/kg were toxic to the liver. Interesting to note is that the UDS with CP was negative in this publication.</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>3-day repeat dose oral comet assay in liver and blood of male SD rats following the JaCVAM protocol Top dose of 1 mg/kg/day was considered the MTD</td>
<td>Increase in DNA damage in the liver is associated with histopathological findings</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>Roche EMS</td>
<td>JaCVAM Protocol, with the exception that PI instead of SYBR Gold was used for DNA staining</td>
<td>Pos (Liver, stomach)</td>
<td>Data from the same animals are available to compare with Novartis</td>
</tr>
<tr>
<td>MMS</td>
<td></td>
<td>Pos (Liver, jejunum, bladder - only 2 dose levels); blood (day 22)</td>
<td>High dose not the MTD for an acute treatment but 28 d treatment</td>
</tr>
<tr>
<td>Temozolomide</td>
<td></td>
<td>Pos (Liver, jejunum, blood)</td>
<td></td>
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<tr>
<td>AZT</td>
<td></td>
<td>Pos(Liver, stomach, blood)</td>
<td>Treatment over 7d</td>
</tr>
<tr>
<td>Japanese lab</td>
<td>JaCVAM final protocol with 3 different strains of rat</td>
<td>Positive (liver and urinary bladder) at similar doses in all strains, male and female</td>
<td>Positive</td>
</tr>
<tr>
<td>(not named)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>